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14. ABSTRACT

Immunotherapy is rapidly becoming accepted as an effective way to treat human cancer. A large proportion of B-cell malignancies express the product of their re-arranged immunoglobulin (IG) genes on the cell surface. Surface-expressed IG constitutes a de facto tumor-specific target antigen. Here we report that two B-cell tumor cell lines, JVM13 and Mec1, express surface IG. Therefore we cloned and sequenced the cDNA encoding the variable portions of these IGs. Specifically, we identified the CDR3 sequences of the heavy and light chain variable portions for each IG, which constitute the molecular target. We have now engineered the following CDR3 sequences "ARSQGVLTAIDY"/"QQYYSIPYT" for the Mec1 heavy and light chains respectively and "ASSYYDILTGYLYYYFDY "/"SSYTSSSTLMI" for the JVM13 heavy and light chains, into a model antibody 4D5 (see figures 1-5 in the report). The "Tomlinson" human antibody phage library will be used to pan for antibodies that bind these target CDR3s and not the parent 4D5 antibody. To confirm the utility of the Tomlinson library and gain experience with handling this complex selection system we have confirmed selection of phage to a test antigen. Successful expression will lead directly to the selection of CDR3-specific antibody-encoding phage: from which we will make our final immunotherapeutic agents.

15. SUBJECT TERMS
Immunotherapy, B-Cell tumor, cancer

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INTRODUCTION:
In the US the incidence of newly diagnosed B-cell leukemias and lymphomas amounts to approximately 100,000 cases per year. This is matched by approximately 40,000 deaths in the same population. While current treatments are improving, side effects from traditional chemotherapy can be debilitating often causing significant life-long morbidity. Compared with chemotherapy, immunotherapy promises similar or better results with far fewer side effects. A potential treatment for individuals who display immunoglobulin protein on the surface of their malignant cells, is targeted immunotherapy that is cytotoxic for only tumor cells. But such immunotherapy strategies are usually time-consuming and complex to organize. To address this limitation we are attempting to speed and simplify the process of producing antibodies to tumor-expressed surface immunoglobulin. Additionally, once we produce anti-tumor antibodies we plan on 'arming' them with toxins or producing CAR T-cells. In this way we will generate novel immunotherapeutic treatments for B-cell malignancies.

BODY:
Statement/Scope of Work (Sow)

Task 1. (Immediately below is the SOW for Task 1 submitted in the original proposal. Below that are the results and data from Task 1).
Using DNA molecular biology software, convert cDNA sequence data into candidate IGHV and IGLV gene sequences from patient sequence data and from model cell line that will serve as target cell in Task 5. Perform analysis to assign candidate sequence into specific immunoglobulin family. Design synthetic genes corresponding to variable regions of heavy and light chains with restriction enzyme sites at each end to allow cloning into the invivogen vectors.

1A. Obtain cDNA sequence data from model B-cell line as model target line for experiments described in Task 5 - month 1-2.
1B. convert raw sequence data candidate IGHV and IGLV - month 1-2
1C. establish immunoglobulin family of candidate sequence - month 1-2
1D. design synthetic genes for antibodies month 1-2

Progress: all elements of Task 1 were accomplished. We purchased two cell lines from ATCC (www.atcc.org): Mec1 and JVM13.

Task 1A
The cDNA sequence for Mec1 is:
VH:
GTCCTGGACCTCCTGTGCAAGAACATGAAACATCTGTGGTTCCTCCTTCTCC
TGGTGGCAGCTCCCAGATGGGTCCTGTCCCAGGTGCTTCTCCAGGAGTCGGCC
CAGGACTGGTGAAGCCTTCGGAGACCCTGTCCCTCACCTGCGCTGTCTCTAGTGG
CTCCATCAGTAGTTATTACTGGAGCTGGATCCGCCAGCCCCCAGGGAAGGGACTG
CAGTGGATTGGCTATATGTATAAAAGTGGGAGCACCAACTACAATCCCTCTCTCAA
GAGTCGAGTCACCATATCAGTAGACACGTCCAAGAACCAGTTCTCCCTGAAGTTGA

GCTCTGTGACCGCTGCGGACACGGCCATGTATTACTGTGCGAGAAGTCAAGGGGT
GCTGACTGCTATTGACTACTGGGGCCAGGGAATCCTGGTCACCGTCTCCTCAGGT
AAGAAG

VL:

ATGGTGTTGCAGACCCAGGTCTTCATTTCTCTGTTGCTCTGGATCTCTGGTG
CCTACGGGGACATCGTGATGACCCAGTCTCCAGACTCCCTGGCTGTGTCTCTGGG
CGAGAGGGCCACCATCAACTGCAAGTCCAGCCAGAGTGTTTTATACAGCTCCAAC
AATAAGAACTACTTAGCTTGGTACCAGCAGAAACCAGGACAGCCTCCTAAGCTGCT
CATTGACTGGGCATCTACCCGGGAATCCGGGGTCCCTGACCGAATCAGTGGCAGC
GGGTCTGGGACAGACTTCACTCTCACCATCAGCAGCCTGCAGGCTGAAGATGTGG
CAGTTTATTACTGTCAGCAGTATTATAGTATTCCGTACACTTTTGGCCAGGGGACCA
AGGTGGAGATAAGAAACGTAAGAAG

The cDNA sequence of JVM13 is:

VH:

GTCCTGGACCTCCTGTGCAAGAACATGAAACATCTGTGGTTCTTCCTTCTCC
TGGTGGCAGCTCCCAGATGGGTCCTGTCCCAGGTGCAGCTGCAGGAGTCGGGCC
CAGGACTGGTGAAGCCTTCGGAGACCCTGTCCCTCACCTGCACTGTCTCTGGTGG
CTCCATCAGTAGTTACTATTGGAGTTGGATCCGGCAGTCCCCAGGGAAGGGACTG
GAGTGGATTGGGTATATCTATTATAGTGGGAGCACCAACTACAACCCCTCCCTCAA
GAGTCGACTCACCATATCAGTAGACACGTCCAAGAACCACTTCTCCCTGAAGCTGA
GCTCTGTGACCGCTGCGGACACGGCCATGCTGTATTACTGTGCGAGTTCGTATTA
CGATATTTTGACTGGTTACCTCTACTACTACTTTGACTACTGGGGCCAGGGAACCC
CGGTCACCGTCTCCTCAGGTAAGAAGGGCGAATTC

VL:

GAATTCGCCCTTATGGCCTGGGCTCTGCTGCTCCTCACCCTCCTCACTCAG
GGCACAGGGTCCTGGGCCCAGTCTGCCCTGACTCAGCCTGCCTCCGTGTCTGGG
TCTCCTGGACAGTCGATCACCATCTCCTGCACTGGAACCAGCAGTGACGTTGGTG
GTTATAACTATGTCTCCTGGTACCAACAACACCCAGGCAAAGCCCCCAAACTCATG
ATTTATGATGTCAGTAATCGGCCCTCAGGGGTTTCTAATCGCTTCTCTGGCTCCAA
GTCTGGCAACACGGCCTCCCTGACCATCTCTGGGCTCCAGGCTGAGGACGAGGC
TGATTATTATTGCAGCTCATATACAAGCAGCAGCACTCTCATGATATTCGGCGGAG
GGACCAAGAAGGGCGAATTCCTCACCGTCCTAGAAGGGCGAATTC

Task 1B

The amino acid sequence for Mec1 IGHV and IGLV is:

VH:

DLLCKNMKHLWFLLLLVAAPRWVLSQVLLQESGPGLVKPSETLSLTCAVSSGSI

SSYYWSWIRQPPGKGLQWIGYMYKSGSTNYNPSLKSRVTISVDTSKNQFSLKLSSVTA
ADTAMYYCARSQGVLTAIDYWGQGILVTVSSGKKGEF

VL:
MVLQTQVFISLLLWISGAYGDIVMTQSPDSLAVSLGERATINCKSSQSVLYSSNN
KNYLAWYQQKPGQPPKLLIDWASTRESGVPDRISGSGSGTDFTLTISSLQAEDVAVYY
CQQYYSIPYTFGQGTKVEIKRKKGEF

The amino acid sequence for JVM13 IGHV and IGLV is:

VH:
VLDLLCKNMKHLWFFLLLVAAPRWVLSQVQLQESGPGLVKPSETLSLTCTVSG
GSISSYYWSWIRQSPGKGLEWIGYIYYSGSTNYNPSLKSRLTISVDTSKNHFSLKLSSV
TAADTAMLYYCASSYYDILTGYLYYYFDYWGQGTPVTVSSGKKGEF

VL:
EFALMAWALLLLTLLTQGTGSWAQSALTQPASVSGSPGQSITISCTGTSSDVGG
YNYVSWYQQHPGKAPKLMIYDVSNRPSGVSNRFSGSKSGNTASLTISGLQAEDEADY
YCSSYTSSSTLMIFGGGTKKGEFLTVLEGRI

Task 1C
The immunoglobulin family for Mec1 IG is:

IGH-V4-59
IGL-V4-01

The immunoglobulin family for JVM13 IG is:

IGH-V4-59
IGL-V2-14

Task 1D
Synthetic genes are as follows:

4D5-7

CATATGAAAAAGACAGCTATCGCGATTGCTGTGGCACTGGCTGGTTTCGCTACCGT
AGCGCAGGCCGATTACAAGGATGACGACGATAAGGAGGTTCAGCTGGTGGAGTCT
GGCGGTGGCCTGGTCCAGCCAGGGGGCTCACTCCGTTTGTCCTGTGCCGCTTCT
GGCTTCAACATTAAAGACACCTATATACACTGGGTGCGTCAGGCCCCGGGTAAGG
GCCTGGAATGGGTTGCAAGGATTTATCCTACGAATGGTTATACTAGATATGCCGAT
AGCGTCAAGGGCCGTTTCACTATAAGCGCAGACACATCCAAAAACACAGCCTACCT
GCAGATGAACAGCCTGCGCGCTGAGGACACAGCCGTCTATTATTGTTCTAGATGG

GGAGGGGACGGCTTCTATGCTATGGACTACTGGGGTCAAGGAACCCTGGTCACC
GTCTCCTCAGGCGGAGGCGGATCCGGTGGTGGCGGCTCTGGTGGTGGCGGCAG
CGATATCCAGATGACCCAGTCCCCGAGCTCCCTGTCCGCCTCTGTGGGCGATAGG
GTCACCATCACATGCCGTGCCAGTCAGGATGTGAATACTGCTGTAGCCTGGTATCA
ACAGAAACCAGGAAAAGCTCCGAAACTACTGATTTACTCGGCATCCTTCCTCGAGT
CTGGCGTCCCTTCTCGCTTCTCTGGATCCAGATCTGGGACGGATTTCACTCTGACC
ATCAGCAGTCTGCAGCCGGAAGACTTCGCAACTTATTACTGTCAGCAACATTATAC
TACGCCTCCCACGTTCGGACAGGGTACCAAGGTGGAGATCAAATGAGAATTC

Mec1

CATATGAAAAAGACAGCTATCGCGATTGCTGTGGCACTGGCTGGTTTCGCTACCGT
AGCGCAGGCCGATTACAAGGATGACGACGATAAGCAGGTTCAGCTCCAGGAATCT
GGTCCGGGTCTGGTTAAACCGTCTGAAACCCTGTCTCTGACCTGCGCGGTTTCTTC
TGGTTCTATCTCTTCTTACTACTGGTCTTGGATCCGTCAGCCGCCGGGTAAAGGTC
TGCAGTGGATCGGTTACATGTACAAATCTGGTTCTACCAACTACAACCCGTCTCTG
AAATCTCGTGTTACCATCTCTGTTGACACCTCTAAAAACCAGTTCTCTCTGAAACTG
TCTTCTGTTACCGCGGCGGACACCGCGATGTACTACTGCGCGCGTTCTCAGGGTG
TTCTGACCGCGATCGACTACTGGGGTCAGGGTATCCTGGTTACCGTTTCTTCTGGT
GGTGGTGGTTCTGGTGGTGGTGGTTCTGGTGGTGGTGGTTCTGACATTGTAATGA
CCCAGAGCCCGGATTCACTCGCGGTCAGTCTCGGCGAACGAGCTACCATCAACTG
CAAATCTTCTCAGTCTGTTCTGTACTCTTCTAACAACAAAAACTACCTGGCGTGGTA
CCAGCAGAAACCGGGTCAGCCGCCGAAACTGCTGATCGACTGGGCGTCTACCCG
TGAATCTGGTGTTCCGGACCGTATCTCTGGTTCTGGTTCTGGTACCGACTTCACCC
TGACCATCTCTTCTCTGCAGGCGGAAGACGTTGCGGTTTACTACTGCCAGCAGTAC
TACTCTATCCCGTACACCTTCGGTCAGGGTACCAAAGTTGAAATCAAATGAGAATT
C

JVM13

CATATGAAAAAGACAGCTATCGCGATTGCTGTGGCACTGGCTGGTTTCGCTACCGT
AGCGCAGGCCGATTACAAGGATGACGACGATAAGCAGGTTCAGCTTCAGGAATCT
GGTCCGGGTCTGGTTAAACCGTCTGAAACCCTGTCTCTGACCTGCACCGTTTCTG
GTGGTTCTATCTCTTCTTACTACTGGTCTTGGATCCGTCAGTCTCCGGGTAAAGGT
CTGGAATGGATCGGTTACATCTACTACTCTGGTTCTACCAACTACAACCCGTCTCT
GAAATCTCGTCTGACCATCTCTGTTGACACGTCTAAAAACCACTTCTCTCTGAAACT
GTCTTCTGTTACCGCGGCGGACACCGCGATGCTGTACTACTGCGCGTCTTCTTACT
ACGACATCCTGACCGGTTACCTGTACTACTACTTCGACTACTGGGGTCAGGGTACC
CCGGTTACCGTTTCTTCTGGTGGTGGTGGTTCTGGTGGTGGTGGTTCTGGTGGTG
GTGGTTCTCAGTCTGCGCTGACCCAGCCGGCGTCTGTTTCTGGTTCCCCGGGTCA
GTCTATCACCATCTCTTGCACTGGTACCTCTTCTGACGTTGGTGGTTACAACTACGT
TTCTTGGTACCAGCAGCACCCGGGTAAAGCGCCGAAACTGATGATCTACGACGTT
TCTAACCGTCCGTCTGGTGTTTCTAACCGTTTCTCTGGTTCTAAATCTGGTAACACC

GCGTCTCTGACCATCTCTGGTCTGCAGGCGGAAGACGAAGCGGACTACTACTGCT
CTTCTTACACCTCTTCTTCTACCCTGATGATCTTCGGTGGTGGTACCAAAAAAGGTG
AGTTCCTGACCGTTCTGTAATGAGAATTC

Task 2. (Immediately below is the SOW for Task 2 submitted in the original proposal. Below that are the results and data from Task 2).
Verify clones via DNA sequencing. Transfer clones into expression vectors. Begin expression of cloned antibodies. Purify cloned antibodies using protein G column.

2A. Verify clones via DNA sequencing - month 3
2B. Subclone IGHV and IGLV into expression vectors - month 4
2 C. Express cloned antibodies into supernatants of CHO cultured cells - month 5-6.
2D. Purify expressed antibody using protein G affinity chromatography - month 7-8.

Progress: all elements of Task 2 have been accomplished. However, we changed our approach from expression in mammalian cells and instead have expressed cloned antibodies in bacterial cells.

Task 2A. We cloned the variable portions of the heavy and light chains of the Mec1 IG and the JVM13 IG. Those sequences are provided immediately below.

Mec1 DNA sequence: variable heavy chain linker and then variable light chain

CATATGAAAAAGACAGCTATCGCGATTGCTGTGGCACTGGCTGGTTTCGCTACCGT
AGCGCAGGCCGATTACAAGGATGACGACGATAAGCAGGTTCAGCTCCAGGAATCT
GGTCCGGGTCTGGTTAAACCGTCTGAAACCCTGTCTCTGACCTGCGCGGTTTCTTC
TGGTTCTATCTCTTCTTACTACTGGTCTTGGATCCGTCAGCCGCCGGGTAAAGGTC
TGCAGTGGATCGGTTACATGTACAAATCTGGTTCTACCAACTACAACCCGTCTCTG
AAATCTCGTGTTACCATCTCTGTTGACACCTCTAAAAACCAGTTCTCTCTGAAACTG
TCTTCTGTTACCGCGGCGGACACCGCGATGTACTACTGCGCGCGTTCTCAGGGTG
TTCTGACCGCGATCGACTACTGGGGTCAGGGTATCCTGGTTACCGTTTCTTCTGGT
GGTGGTGGTTCTGGTGGTGGTGGTTCTGGTGGTGGTGGTTCTGACATTGTAATGA
CCCAGAGCCCGGATTCACTCGCGGTCAGTCTCGGCGAACGAGCTACCATCAACTG
CAAATCTTCTCAGTCTGTTCTGTACTCTTCTAACAACAAAAACTACCTGGCGTGGTA
CCAGCAGAAACCGGGTCAGCCGCCGAAACTGCTGATCGACTGGGCGTCTACCCG
TGAATCTGGTGTTCCGGACCGTATCTCTGGTTCTGGTTCTGGTACCGACTTCACCC
TGACCATCTCTTCTCTGCAGGCGGAAGACGTTGCGGTTTACTACTGCCAGCAGTAC
TACTCTATCCCGTACACCTTCGGTCAGGGTACCAAAGTTGAAATCAAATGAGAATT
C

JVM13 DNA sequence: variable heavy chain linker and then variable light chain

CATATGAAAAAGACAGCTATCGCGATTGCTGTGGCACTGGCTGGTTTCGCTACCGT
AGCGCAGGCCGATTACAAGGATGACGACGATAAGCAGGTTCAGCTTCAGGAATCT
GGTCCGGGTCTGGTTAAACCGTCTGAAACCCTGTCTCTGACCTGCACCGTTTCTG
GTGGTTCTATCTCTTCTTACTACTGGTCTTGGATCCGTCAGTCTCCGGGTAAAGGT
CTGGAATGGATCGGTTACATCTACTACTCTGGTTCTACCAACTACAACCCGTCTCT
GAAATCTCGTCTGACCATCTCTGTTGACACGTCTAAAAACCACTTCTCTCTGAAACT
GTCTTCTGTTACCGCGGCGGACACCGCGATGCTGTACTACTGCGCGTCTTCTTACT
ACGACATCCTGACCGGTTACCTGTACTACTACTTCGACTACTGGGGTCAGGGTACC
CCGGTTACCGTTTCTTCTGGTGGTGGTGGTTCTGGTGGTGGTGGTTCTGGTGGTG
GTGGTTCTCAGTCTGCGCTGACCCAGCCGGCGTCTGTTTCTGGTTCCCCGGGTCA
GTCTATCACCATCTCTTGCACTGGTACCTCTTCTGACGTTGGTGGTTACAACTACGT
TTCTTGGTACCAGCAGCACCCGGGTAAAGCGCCGAAACTGATGATCTACGACGTT
TCTAACCGTCCGTCTGGTGTTTCTAACCGTTTCTCTGGTTCTAAATCTGGTAACACC
GCGTCTCTGACCATCTCTGGTCTGCAGGCGGAAGACGAAGCGGACTACTACTGCT
CTTCTTACACCTCTTCTTCTACCCTGATGATCTTCGGTGGTGGTACCAAAAAAGGTG
AGTTCCTGACCGTTCTGTAATGAGAATTC

Task 2B. IG sequences from both cell lines were subcloned into bacterial expression vectors. An OmpA signal and Flag-tag sequences were engineered just before the IG sequences. An illustration of this is provided in Figures 1-5.

Task 2C. Expressed clones produced antibodies in the periplasm preparations of bacteria. The presence of the antibodies was confirmed with anti-Flag western blots and these are provided in Figure 6.

Task 2D. Antibodies were purified using a combination of Flag-tag affinity column chromatography and size exclusion chromatography. Examples are shown in Figure 7.

Task 3. Immediately below is the SOW for Task 3 submitted in the original proposal. Below that are the results and data from Task 3.
Selection of phage that bind to the purified candidate antibody and not to irrelevant antibodies. Phage are from single chain Fv library. Sequence phage that bind to candidate antibodies.

3A. Select binders from phage library - month 9.
3B. Enrich for high affinity binders via multiple rounds of selection - month 10-11.
3C. Sequence phage at the end of three rounds of panning and verify enrichment and sequence as a single chain Fv - month 12-13.

Progress: No element of Task 3 has been completed yet. We are few months behind in schedule. However, when it was clear that antibody purification lagged we used the phage library to select for antibodies that bound to a model antigen. This gave Ms Weiss practice with the system and allowed us to confirm the functionality of the 'Tomlinson' library.

The following tasks have not begun.

Task 4. Convert single chain Fv from library into a therapeutic antibody

4A. Convert single chain Fv into immunotoxin - month 14
4B. Convert single chain Fv into a full-length antibody - month 15-16
4C. Convert single chain Fv into a Chimeric antigen receptor- month 17- 20

Task 5. Conduct preliminary experiments in tissue culture to characterize the cytotoxic activity of candidate immunotoxins, full-length antibodies and chimeric antigen receptor constructs - months 21-24.

KEY RESEARCH ACCOMPLISHMENTS:

- Purchased two B-cell tumor cell lines, Mec1 and JVM13
- Obtained 'Tomlinson' human antibody phage library from Dr Mitchell Ho
- Determined that both lines express surface immunoglobulin (IG)
- Used PCR and cDNA cloning techniques to obtain IG sequence data
- Established that Mec1 IG from the XXX family
- Established that JVM13IG is from the YYY family
- Synthesized the 4D5 single chain Fv as a negative control antibody
- Determined the CDR3 sequences from the IG heavy and light chains
- Constructed hybrid antibody genes by inserting CDR3 into 4D5
- Constructed expression plasmids with OmpA and Flag-tag sequences
- Expressed single chain Fvs of 5 antibodies to the perimplasm of E coli
- Purified antibodies via Flag affinity resins and size exclusion chromatography
- Verified utility of 'Tomlinson' phage library using model antigen
  Selected antibody from Tomlinson library via antigen panning

REPORTABLE OUTCOMES:

The PI attended IBC Antibody Engineering Meeting, San Diego, CA in Dec 2012.
Ms Weiss attended the “BIOTRAC8: Immunochemistry and Monoclonal Antibody Production” course from 7/22/2013-7/26/2013.
Ms Weiss has prepared a draft manuscript describing her results (Attached as appendix 1).

CONCLUSION:

Excellent progress has been made toward completing our stated goals and tasks. We have cloned, sequenced and engineered the variable portions of two surface immunoglobulin molecules. These immunoglobulin molecules were detected on the surface of the Mec1 and JVM13 cell lines. CDR3 sequences from these immunoglobulins were identified and then engineered into the model 4D5 antibody as 'replacement inserts'. CDR3 inserts were engineered into both the heavy and light chains. The 4D5-Mec1 and 4D5-JVM13 antibodies are now being probed with our antibody phage library to select binders that react only with the CDR3 portion of each antibody. Separately, the utility of the phage library was confirmed.

REFERENCES:
None

APPENDIX 1:

CDR3-directed Immunotherapy

Authors: Emily Weiss, Bob Sarnovsky, Antonella Antignani, and David FitzGerald

While standard chemotherapeutic protocols can significantly decrease tumor burden and prolong life expectancy, they are unable to cure many patients diagnosed with B cell malignancies; therefore, there exists a need for the research and development of alternative treatment approaches. In the area of cancer research, there has been a rising interest in recruiting the patient's own immune system to target existing tumors. This type of immunotherapy can involve methods such as immunization against tumor cells or delivery of a toxic agent through the use of an antibody targeting a tumor specific antigen (TSA). Unique sequences located in the immunoglobulin molecules on the cell surface of B cells represent one such TSA.

Immunoglobulins expressed on the cell surface of B-lymphocytes undergo a complex process known as hypersomatic mutation when presented with antigen. It involves the rearrangement of immunoglobulin gene segments such that the end protein can efficiently bind a specific antigen, allowing for a proliferation and survival signal to be sent into the B cell. The result is a population of mature B cells displaying identical immunoglobulin, which recognize a single target antigen. This specificity can be attributed to the Complementary Determining Regions (CDRs) located in the variable regions of both heavy and light chains. Out of six CDRs, it is believed that the CDR3 exhibits the most diversity and therefore most unique to its target antigen. B cell malignancies are clonal cancers and therefore express identical immunoglobulin on their cell surface. It follows then, that the malignant B cell CDR3s should make a reliable TSA.

Here we investigate a novel method in the treatment of B cell cancers through a rapid procession of cloning and phage display to isolate antibodies to the variable regions and CDR3s of immunoglobulin. These antibodies can then be used as targeted delivery systems for cytotoxic agents, such as an antibody drug conjugate or a cytotoxic T cell expressing a chimeric antigen receptor (CAR).

As outlined in the project narrative, we have chosen to study two tumor cell lines and cloned the variable regions of both heavy and light chains. The tumor cell lines selected were MEC1 and JVM13, a CLL and PLL cell line, respectively (SU-DHL-6 was not used due to loss of viability only weeks after being reintroduced into media and standard culture conditions). Total RNA was isolated from cells. Using 5ug of isolated total RNA, reverse transcription was performed using SuperScriptTM III Reverse Transcriptase kit from Invitrogen. This yielded single stranded cDNA to be used as a template in IgH and IgL cloning. Primers purchased from BIOMED-2 in combination with AmpliTaq Gold master mix were used in the PCR reactions, primers can be seen below in Fig 1-3.

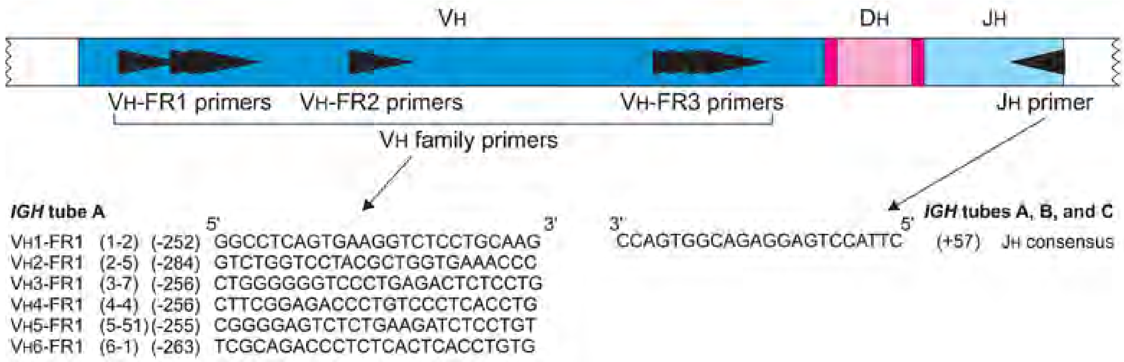


Figure 1. Primer mix used in IGH cloning reactions. (Find concentrations used) of both forward and reverse primer were mixed with 8ul cDNA and 25ul AmpliTaq Master Mix. PCR conditions were as follows: 95^{O}C – 7minutes; 30 cycles: 95^{O}C – 1minute, 55^{O}C – 30seconds, 72^{O}C – 1minute; 72^{O} – 10minutes. Check #1 TA-1

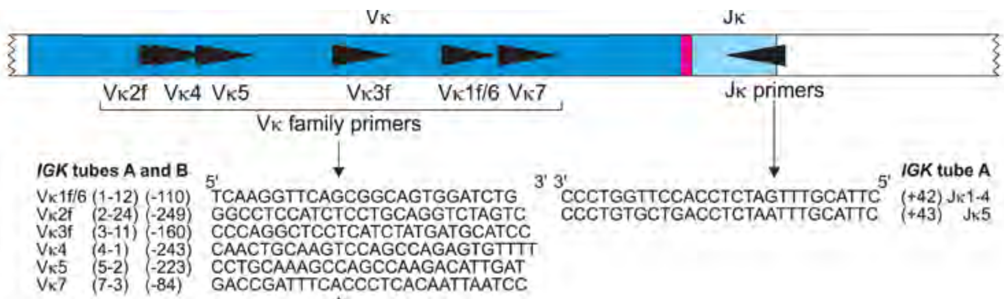


Figure 2. Primer mix used in IGK cloning reactions. 45ul of primer mix was mixed with 5ul cDNA and 0.5ul pure AmpliTaq enzyme. PCR prog #36 IGL (look up) Cloning was only performed for MEC1.

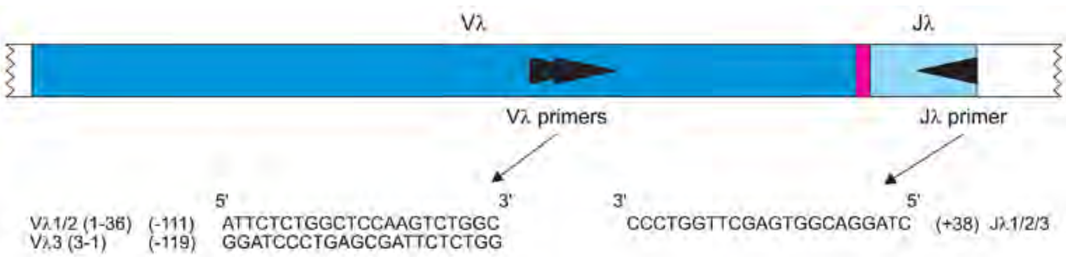


Figure 3. Primer mix used in IGL cloning reactions. 45ul of primer mix was mixed with 5ul cDNA and 0.5ul pure AmpliTaq enzyme. PCR prog #36 IGL (look up) Cloning was only performed for JVM13.

PCR sample from each cloning reaction was run on an agarose gel to check for purity and size of product. Cloned segments were then ligated into the TA-TOPO vector (Invitrogen), transformed into competent E. coli cells, and plated on Kanamycin 50mg/ml plates. Bacterial colonies were then grown out and the plasmids isolated using the MiniPrep kit (QIAGEN). Purified plasmids were then sent out to a sequencing lab located at Johns Hopkins University. Sequencing results were queried against the IMGT

database online to check for V-gene rearrangement and location of CDRs. Respective families for heavy and light chains can be viewed in Table 1 below.

Table 1. List of V-gene families based on sequence data. Sequence data was derived from DNA and queried against IGMT database to give respective V-gene families.

	IGH	*IGK*	*IGL*
MEC1	V4-59	V4-1	
JVM13	V4-59		V2-14

Table 1. List of V-gene families based on sequence data.

While the primers from BIOMED-2 were able to clone the majority of the variable region for both heavy and light chain, some sequences were incomplete. Therefore, primers were designed by resident immunologist Evgeny Arons so that the full heavy and light chain variable regions for both MEC1 and JVM13 cell lines could be sequenced. Those primers can be viewed below in Table 2. Sequencing results are reported below in Fig 4.

Table 2. Forward and reverse oligos used in cloning to get full V-region sequences. IGH used in both MEC1 and JVM13 PCR reactions, while IGK was used only for MEC1 and IGL used only for JVM13. All primers listed 5'-3'.

	IGH	IGK	IGL
Forward	AGTCCTGGACCTCCTGTGCAA	CGGGCCGTTTGCATTGTGAACT	ATGGCCTGGGCTCTGCTGCTCC
Reverse	TACATGGCCGTGTCCGCAGC	ATCTCCACCTTGGTCCCCTGG	CTTGGTCCCTCCGCCGAATATCAT

a.

QVLLQESGPGLVKPSETLSLTCAVSSGSISSYYWSWIRQPPGKGLQWIGYMYKSGSTNYNPSLK
SRVTISVDTSKNQFSLKLSSVTAADTAMYYCARSQGVLTAIDYWGQGILVTVSS

b.

DIVMTQSPDSLAVSLGERATINCKSSQSVLYSSNNKNYLAWYQQKPGQPPKLLIDWASTRESGV
PDRISGSGSGTDFTLTISSLQAEDVAVYYCQQYYSIPYTFGQGTKVEIK

c.

QVQLQESGPGLVKPSETLSLTCTVSGGSISSYYWSWIRQSPGKGLEWIGYIYYSGSTNYNPSLK
SRLTISVDTSKNHFSLKLSSVTAADTALYYCASSYYDILTGYLYYYFDYWGQGTPV

d.

QSALTQPASVSGSPGQSITISCTGTSSDVGGYNYVSWYQQHPGKAPKLMIYDVSNRPSGVSNR
FSGSKSGNTASLTISGLQAEDEADYYCSSYTSSSTLMIFGGGTK

Figure 4. Protein sequences for V-region of MEC1 and JVM13 heavy and light chains. Highlighted sequence regions are CDR1 (green), CDR2 (blue), and CDR3 (orange). (a) MEC1 IgH; (b) MEC1 IgK; (c) JVM13 IgH; (d) JVM13 IgL.

Once the sequences for MEC1 and JVM13 heavy and light chains were known, 3 codon-optimized synthetic genes were ordered from Blue Heron Biotechnologies: a full 4D5 antibody copied from Genentech, a full MEC1 and a full JVM13. From these synthetic genes, five proteins were made in order to use in phage display. The five proteins are a full 4D5 antibody, full MEC1 and JVM13 antibodies, and the full 4D5 antibody with the either the CDR3s for heavy and light chains from MEC1 or JVM13 swapped for the 4D5 CDR3s (4D5MEC1 and 4D5JVM13, respectively). The CDR3s for MEC1 and JVM13 were cloned into the full 4D5 using mini constructs. A schematic of the five proteins can be seen in Fig 5.

Figure 5. Single-chain variable fragments produced for phage display.

Minigenes for antibodies in Fig5 were cloned into an immunotoxin expression vector (one that was readily available in our lab) with a T7 promoter. These minigenes included an OmpA signal and FLAG tag, which when translated to protein are present at the N-terminus. Once the proteins are made and directed to the periplasm through the OmpA signal, the cells are lysed and the soluble protein that was extracted was run through an Anti-DYKDDDDK G1 (Anti-Flag) Affinity Resin (GenScript). The bound scFvs were then eluted and purified in soluble form.

All five scFvs will be used in panning with the Tomlinson I+J phage libraries. Ultimately, we want phage expressing randomized scFvs to bind only the CDR3s of MEC1 and JVM13, meaning that after the panning process, selected phage should only bind the 4D5MEC1 and 4D5JVM13 and not the full 4D5. However, although both Tomlinson libraries contain great diversity ($I=1.47 \times 10^8$ and $J=1.37 \times 10^8$), there could possibly not be a scFv expressing phage that will bind to only the CDR3s. Therefore, panning will also be done on the full MEC1 and JVM13 scFvs.

SUPPORTING DATA:

Fig 1. humAb4D5-7 wt OmpA flag

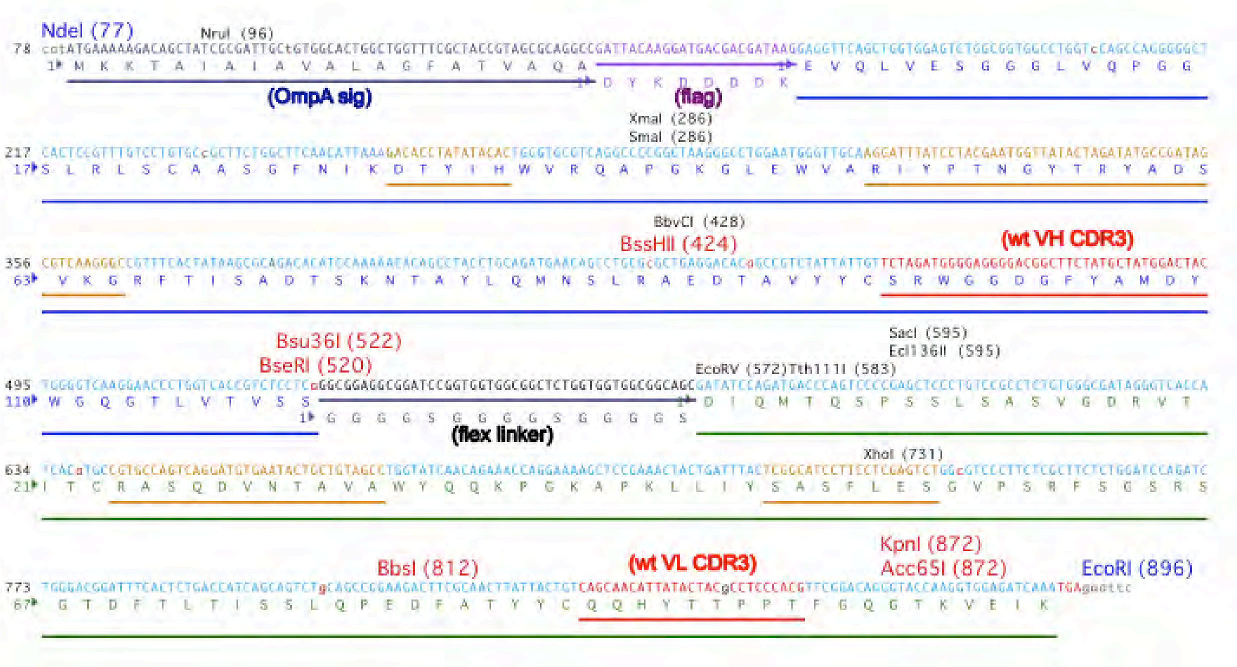


Fig 2

humAb4D5-7 MEC1 OmpA flag

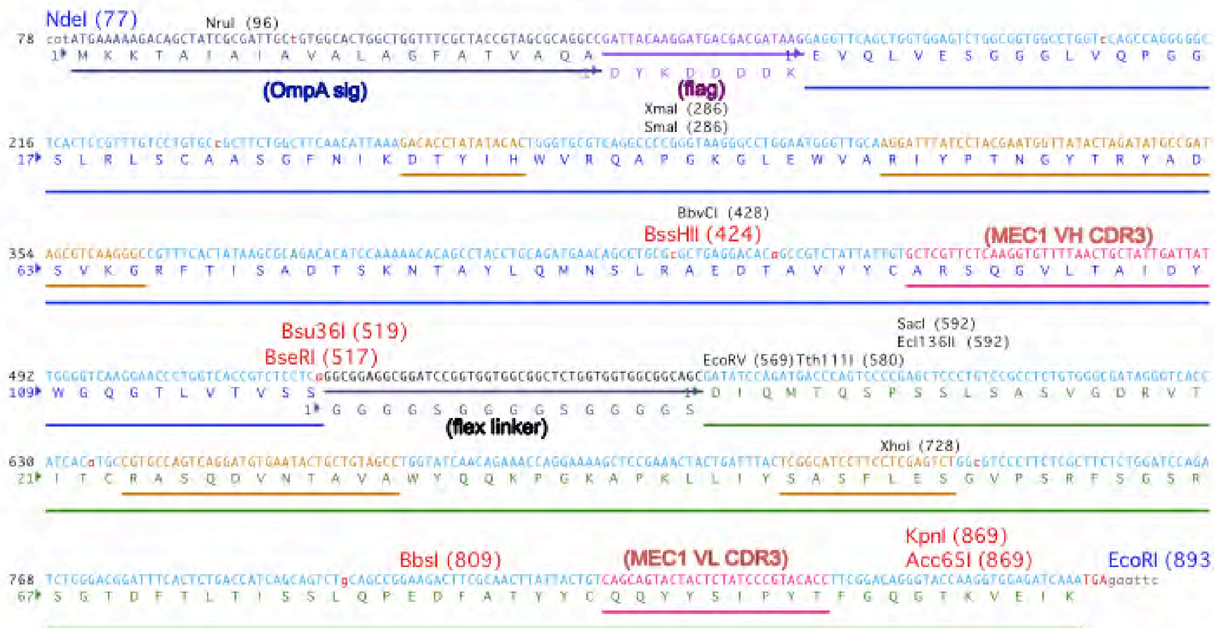


Fig 3

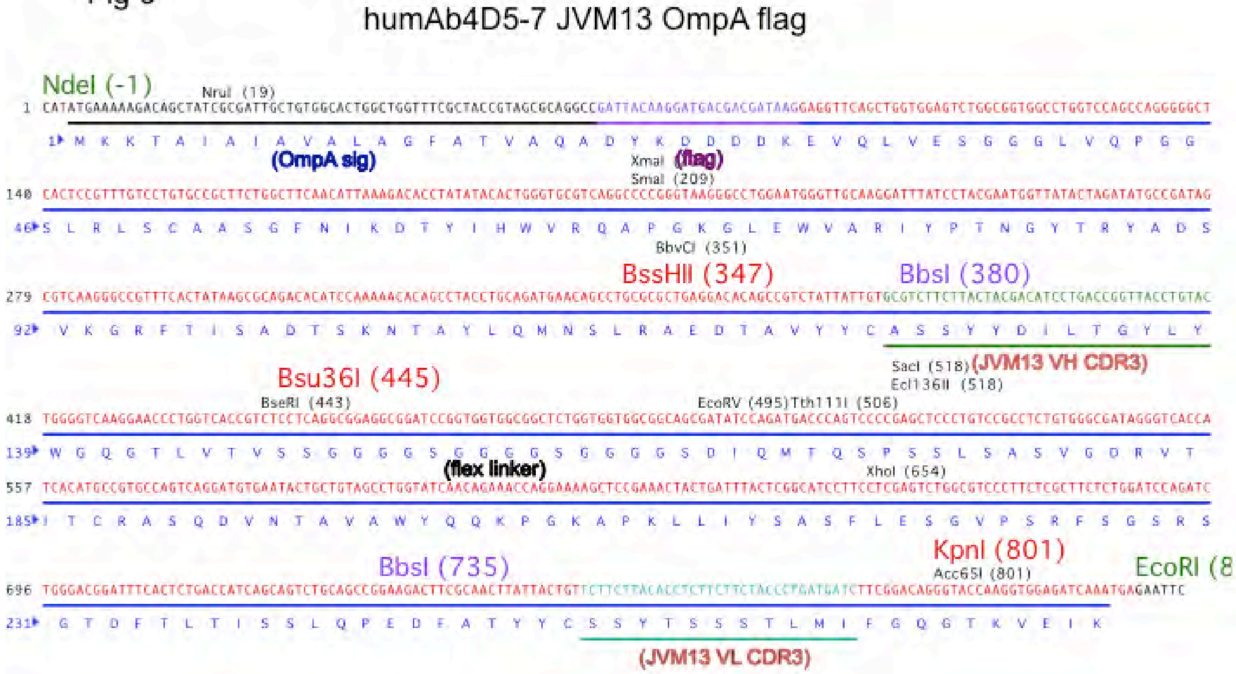

humAb4D5-7 JVM13 OmpA flag
NdeI (-1)
NruI (19)
(OmpA sig)
XmaI
SmaI (209)
(flag)
BbvCI (351)
BssHII (347)
BbsI (380)
(JVM13 VH CDR3)
SacI (518)
Ecl136II (518)
Bsu36I (445)
BseRI (443)
EcoRV (495)
Tth111I (506)
(flex linker)
XhoI (654)
BbsI (735)
KpnI (801)
Acc65I (801)
EcoRI (8
(JVM13 VL CDR3)

Fig 4

Full MEC1 OmpA flag

Fig 5

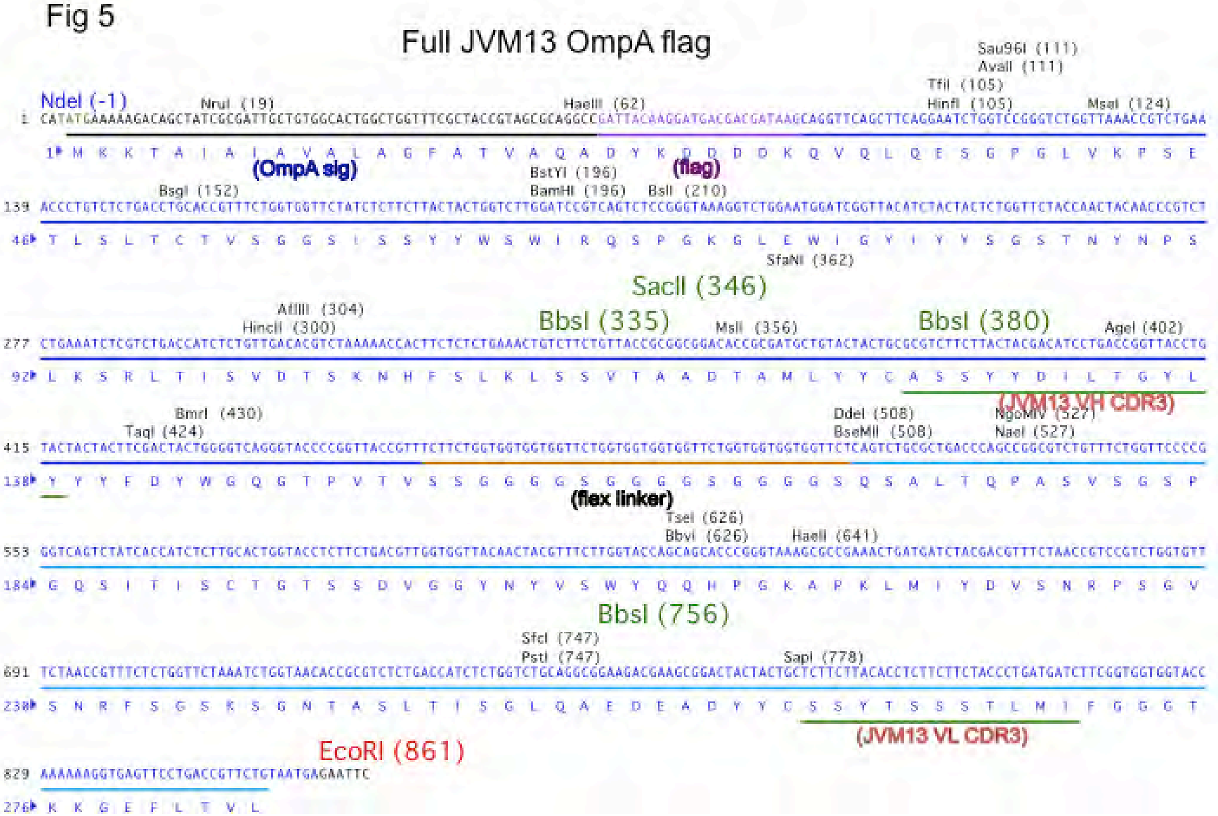
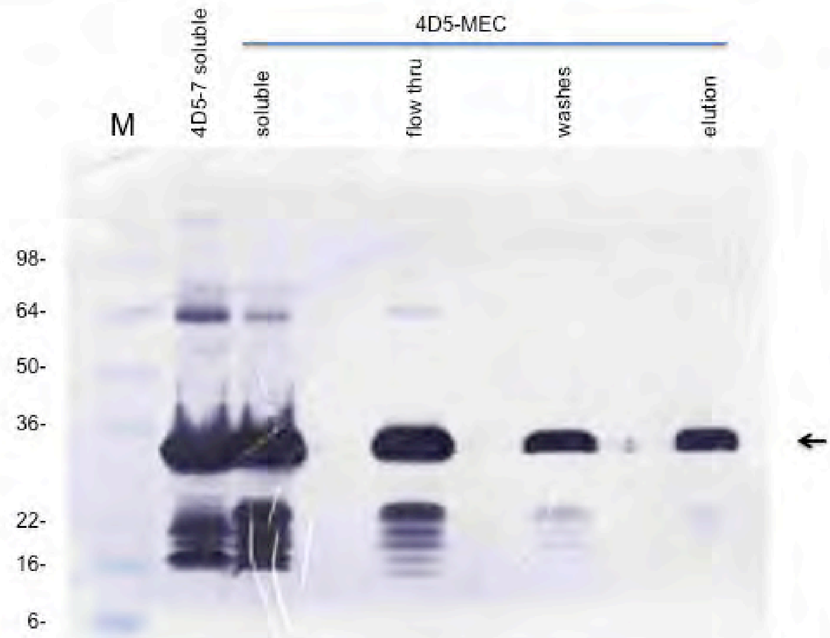

Full JVM13 OmpA flag
NdeI (-1)
NruI (19)
HaeIII (62)
Sau96I (111)
AvaII (111)
TfiI (105)
HinfI (105)
MsaI (124)
(OmpA sig)
(flag)
BsgI (152)
BstYI (196)
BamHI (196)
BslI (210)
SfaNI (362)
SacII (346)
AflIII (304)
HincII (300)
BbsI (335)
MslI (356)
BbsI (380)
AgeI (402)
(JVM13 VH CDR3)
BmrI (430)
TaqI (424)
DdeI (508)
BseMII (508)
NgoMIV (527)
NaeI (527)
(flex linker)
TseI (626)
BbvI (626)
HaeII (641)
BbsI (756)
SfcI (747)
PstI (747)
SapI (778)
(JVM13 VL CDR3)
EcoRI (861)

Fig 6.

Representative western for 4D5MEC anti-FLAG resin purification



(western blot using anti-FLAG antibody

Final 4D5 MEC purified protein (post Superdex 200 size exclusion column)

Fig 7.

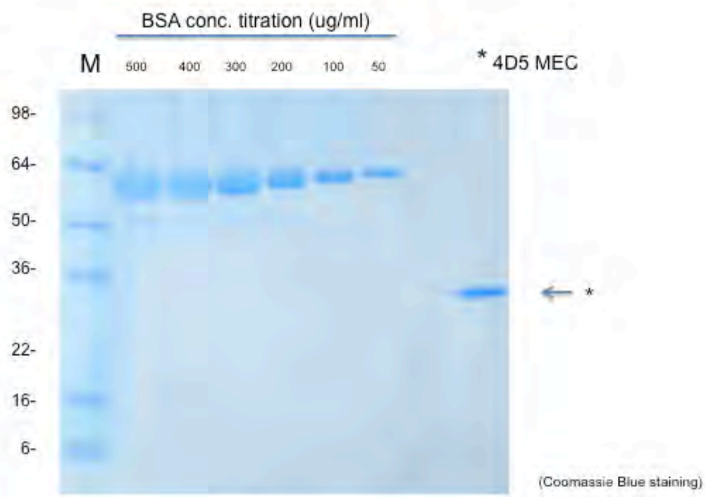


Figure Legends:

Fig 1. Shown is the DNA and protein sequence of the 4D5 single chain Fv antibody incorporated into the OmpA/Flag-tag expression vector. This is the negative control antibody generated to absorb irrelevant Phage.

Fig 2. This is the DNA and protein sequence of the expression vector whereby the CDR3 sequences from the 4D5 antibody have been replaced with the CDR3 sequences from the tumor immunoglobulin displayed on the surface of the Mec1 tumor line.

Fig 3. This is the DNA and protein sequence of the expression vector whereby the CDR3 sequences from the 4D5 antibody have been replaced with the CDR3 sequences from the tumor immunoglobulin displayed on the surface of the JVM13 tumor line.

Fig 4. Shown is the cloned DNA sequence and the translated amino acid sequence of the variable portions of the Mec1 surface immunoglobulin. Both heavy and light chain sequences are supplied.

Fig 5. Shown is the cloned DNA sequence and the translated amino acid sequence of the variable portions of the JVM13 surface immunoglobulin. Both heavy and light chain sequences are supplied.

Fig 6. Representative purification of cloned antibody proteins from the OmpA/Flag-tag expression vector. From left to right, proteins of increasing purity are shown. Detection is via the Flag-tag on the N-terminus of the protein. It is detected with an anti-FLAG antibody. By way of example, the 4D5-Mec1-CDR3 protein is shown. Other proteins purify in a similar way.

Fig 7. Purity of the final product is shown. Decreasing amounts of albumin are shown on the gel for comparing gel loading and estimating protein concentrations. The band to the extreme right is the 4D5-Mec1CDR3 protein that was expressed and purified.